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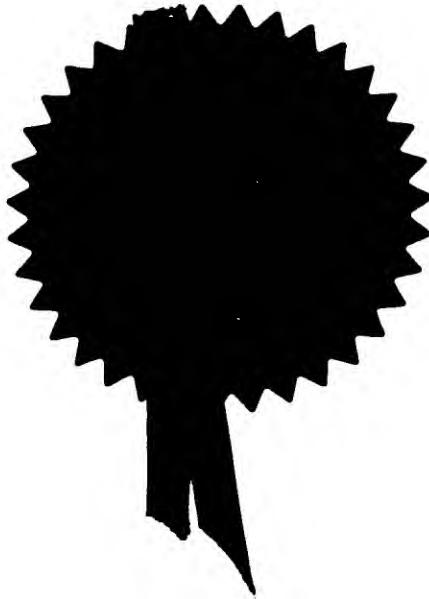
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THERAPEUTIC COMPOUNDS AND METHODS OF
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Therapeutic compounds and methods of making and using the same

The present invention relates to compounds useful in the treatment of disorders associated with abnormal blood glucose levels, particularly in the prevention of phosphorylase-a binding to the glycogen targeting subunit (G_L) of protein phosphatase 1 (PP1). Such compounds are useful for increasing glycogen synthesis and thereby reducing blood glucose levels. The compounds find utility in the treatment of disorders, such as type I and type II diabetes, associated with higher than normal levels of blood glucose (hyperglycaemia).

Most of the adverse physiological consequences in type I and type II diabetes arise from the higher than normal levels of blood glucose. Although high blood glucose levels can be reduced by administration of insulin in type 1 diabetes and by dietary restrictions in the case of type II diabetes, a drug which aids reduction of blood glucose levels would be advantageous in the treatment of these disorders. The liver, which is the main organ regulating glucose homeostasis, is able to store glucose in the form of glycogen and the synthesis of hepatic glycogen from glucose is under the control of hepatic glycogen synthase.

Protein phosphatase 1 is major protein serine/threonine phosphatase in eukaryotic cells, which regulates numerous distinct cellular processes. This is achieved by the interaction of the catalytic subunit of PP1 with a diverse range of targeting subunits that localise PP1C to specific sites within the cell, modulate its activity towards particular substrates and allow its activity to respond to extracellular signals [1,2].

The family of proteins that target PP1 to glycogen and regulate its activity towards the enzymes of glycogen metabolism consists of four members,

G_M /PPP1R3, G_L /PPP1R4, PPP1R5 and PPP1R6/PTG [3-8]. The liver specific glycogen-targeting subunit, G_L is a 33 kDa protein [5,9] that, when bound to PP1, enhances the rate at which the latter dephosphorylates and activates the rate determining enzyme in glycogen synthesis, glycogen synthase, whilst suppressing the rate at which it inactivates glycogen phosphorylase. The stimulation of hepatic glycogenolysis by glucagon (acting via cyclic AMP and PKA) and α -adrenergic agonists (acting via Ca^{2+}) is achieved by activation of phosphorylase kinase respectively, which increases the levels of the active phosphorylated form of glycogen phosphorylase (phosphorylase α). In addition, phosphorylase α binds to G_L and potently inhibits its glycogen synthase phosphatase activity thereby inhibiting glycogen synthesis. Insulin lowers hepatic cMP levels, causing a reduction in the level of phosphorylase α and alleviation of the phosphorylase α -mediated inhibition of the PP1 G_L complex, while the binding of glucose to phosphorylase α , increases the rate at which phosphorylase is inactivated. These mechanisms contribute to the stimulation of glycogen synthesis by insulin and high blood glucose [10]. The inhibition of the PP1 G_L complex by phosphorylase α occurs at nanomolar concentrations and is thought to be via an allosteric mechanism since the K_m for phosphorylase α as a substrate is in the micromolar range [11]. This view is strengthened by the finding that phosphorylase α (but not phosphorylase b) binds directly to G_L in protein blotting experiments [5,9].

Recent studies identified conserved regions between the glycogen targeting subunits G_M /PPP1R3 and G_L /PPP1R4 [5]. A peptide corresponding to one of these regions, G_M 63-75 was shown to bind PP1 and the amino terminal 38 residues of the myofibrillar targeting subunit of PP1 were also

demonstrated to interact with PP1 [12]. The G_M 63-75 peptide, which contains a small motif common to the myofibrillar binding subunit and many other of the PP1 targeting subunits has been crystallised as a complex with PP1 and its structure solved to 2.8 Å resolution [2]. This motif, Lys/Arg-Val/Ile-Xaa-Phe/Trp, which has also been identified by a random peptide library approach [13], is found in all the four glycogen targeting subunit and is located at residues 60-64 of G_L . However, incubation of the PP1 G_L complex purified from hepatic glycogen-protein particles with a PP1-binding peptide from G_M failed to dissociate the PP1- G_L complex [12], even though the peptide abolished the suppression of phosphorylase phosphatase activity conferred on PP1 by association with G_L .

The present invention seeks to provide biological materials and methods which may be useful in the treatment of disorders, especially those such as diabetes type I and type II, associated with higher than normal levels of blood glucose.

According to a first aspect of the invention there is provided the use in medicine of a compound which is capable of blocking the interaction of phosphorylase α with the glycogen-targeting subunit (G_L) of protein phosphatase 1.

Preferably, the compound is for use in the manufacture of a medicament for use in treating disorders associated with higher than normal levels of blood glucose. Preferably the medicament is for use in the treatment of a disorder selected from type I and/or type II diabetes.

Preferably, the compound is a polypeptide comprising the sequence of the C-terminal 16 amino acids of human G_L sequence, or a fragment or variant thereof of which is capable of binding phosphorylase α .

Thus, the sequence may be PEWPSYLGYEKLGPYY, which may be the sequence of the C-terminal 16 amino acids of rat liver G_L .

By "variant" we include the meaning of polypeptides comprising an amino acid sequence which, although not identical to the 16 amino acid sequence, are capable of binding phosphorylase α .

By "fragment" we include the meaning that the polypeptide comprises less than the 16 amino acid sequence mentioned above, but is capable of binding phosphorylase α .

The identification of variants and fragments within the scope of the invention can be carried out using the methods described herein.

Preferably the polypeptide increases the activity of hepatic glycogen synthase.

Polypeptides in which one or more of the amino acid residues are chemically modified, before or after the polypeptide peptide is synthesised, may be used in accordance with the invention, providing that the function of the peptide, namely the blocking of the interaction between G_L and phosphorylase α , remains substantially unchanged. Such modifications include forming salts with acids or bases, especially physiologically acceptable organic or inorganic acids and bases, forming an ester or amide of a terminal carboxyl group, and attaching amino acid

protecting groups such as N-t-butoxycarbonyl. Such modifications may protect the peptide from *in vivo* metabolism.

The peptides may be present as single copies or as multiples, for example tandem repeats. Such tandem or multiple repeats may increase the activity of the polypeptide in blocking the binding of G_L and phosphorylase α .

In a second aspect, the invention provides a pharmaceutical composition comprising an inhibitor compound which is capable of blocking the interaction of phosphorylase α with the glycogen targeting subunit (G_L) of protein phosphatase (PP1), together with a pharmaceutically acceptable excipient or carrier. Preferably the inhibitor compound comprises a polypeptide having the sequence of the C-terminal 16 amino acids of human G_L sequence, or a fragment or variant thereof of which is capable of binding phosphorylase α , for example the 16 amino acid sequence PEWPSYLGYEKLGPELL or a fragment or variant thereof of which is capable of binding phosphorylase α .

In a third aspect, the invention provides a method of identifying an inhibitor compound that is capable of blocking the interaction of phosphorylase α with the glycogen-targeting subunit of PP1 comprising: providing a polypeptide comprising the sequence of the C-terminal 16 amino acids of human G_L sequence, or a fragment or variant thereof of which is capable of binding phosphorylase α , for example the 16 amino acid sequence PEWPSYLGYEKLGPELL, or a fragment or variant thereof which binds phosphorylase α ;

providing a test compound; and

comparing the binding of the polypeptide by phosphorylase α in the presence or absence of the test compound; an inhibitor compound being identified by reduced binding in the presence of the test compound.

The inhibitor compound may be a drug-like compound or lead compound for the development of a drug-like compound. Thus, the method may be a method for identifying a drug-like compound or lead compound for the development of a drug-like compound that is capable of blocking the interaction of phosphorylase α with the glycogen-targeting subunit of PP1.

The term "drug-like compound" is well known to those skilled in the art, and may include the meaning of a compound that has characteristics that may make it suitable for use in medicine, for example as the active ingredient in a medicament. Thus, for example, a drug-like compound may be a molecule that may be synthesised by the techniques of organic chemistry, less preferably by techniques of molecular biology or biochemistry, and is preferably a small molecule, which may be of less than 5000 daltons molecular weight and which may be water-soluble. A drug-like compound may additionally exhibit features of selective interaction with a particular protein or proteins and be bioavailable and/or able to penetrate target cellular membranes, but it will be appreciated that these features are not essential.

The term "lead compound" is similarly well known to those skilled in the art, and may include the meaning that the compound, whilst not itself suitable for use as a drug (for example because it is only weakly potent against its intended target, non-selective in its action, unstable, poorly soluble, difficult to synthesise or has poor bioavailability) may provide a

starting-point for the design of other compounds that may have more desirable characteristics.

The compounds identified in the method may themselves be useful as a drug or they may represent lead compounds for the design and synthesis of more efficacious compounds.

The inhibitor compound may be an antibody or immunoglobulin-type molecule or a fragment thereof, as well known to those skilled in the art. An antibody or immunoglobulin-type molecule or a fragment thereof that cross-reacts/binds with a polypeptide having the sequence of the C-terminal 16 amino acids of human G_L sequence, or a fragment or variant thereof of which is capable of binding phosphorylase α , for example the 16 amino acid sequence PEWPSYLGYEKLGPYY or a fragment or variant thereof of which is capable of binding phosphorylase α may be such an inhibitor compound. An antibody or immunoglobulin-type molecule or a fragment thereof that cross-reacts/binds to G_L may also be such an inhibitor compound. Such antibodies or immunoglobulin-type molecules or fragments thereof may be prepared by methods well known to those skilled in the art.

It will be appreciated that by "phosphorylase α " is included variants, fragments and fusions of phosphorylase α that have interactions or activities which are substantially the same as those of phosphorylase α with G_L but which may be more convenient to use in an assay. For example, a fusion of phosphorylase α may be useful since said fusion may contain a moiety which may allow the fusion to be purified readily. It is preferred that the phosphorylase α is phosphorylase α from rabbit skeletal

muscle. Phosphorylase α may be obtained from Sigma, Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, BH12 4QH.

Preferably, the phosphorylase α is labelled and the binding of phosphorylase α to the polypeptide is determined by measuring the amount of label quantitatively or qualitatively.

Conveniently, the phosphorylase α is labelled with a label selected from digoxigenin, ^{33}P and ^{32}P . Phosphorylase α labelled with ^{32}P or ^{33}P may be obtained by phosphorylation by phosphorylase kinase, as described in Cohen *et al* (1988) *Meth Enzymol* 159, 399-408. Phosphorylase kinase may be obtained from Sigma.

The disruption of the interaction between the said polypeptide and phosphorylase α can be measured *in vitro* using methods well known in the art of biochemistry and include any methods which can be used to assess protein-protein interactions.

The said interaction can also be measured within a cell, for example using the yeast two hybrid system as is well known in the art.

It will be appreciated that screening assays which are capable of high throughput operation will be particularly preferred. Examples may include cell based assays and protein-protein binding assays. An SPA-based (Scintillation Proximity Assay; Amersham International) system may be used. For example, beads comprising scintillant and the polypeptide having the sequence of the C-terminal 16 amino acids of human G_L sequence, or a fragment or variant thereof of which is capable

of binding phosphorylase *a*, for example the 16 amino acid sequence PEWPSYLGYEKLGYYY or a fragment or variant thereof of which is capable of binding phosphorylase *a* may be prepared. The beads may be mixed with a sample comprising ^{32}P - or ^{33}P -labelled phosphorylase *a* and with the test compound. Conveniently this is done in a 96-well format. The plate is then counted using a suitable scintillation counter, using known parameters for ^{32}P SPA assays. Only ^{32}P that is in proximity to the scintillant, i.e. only that bound to the polypeptide, is detected. Variants of such an assay, for example in which the polypeptide is immobilised on the scintillant beads *via* binding to an antibody, may also be used.

Other methods of detecting polypeptide/polypeptide interactions include ultrafiltration with ion spray mass spectroscopy/HPLC methods or other physical and analytical methods. Fluorescence Energy Resonance Transfer (FRET) methods, for example, well known to those skilled in the art, may be used, in which binding of two fluorescent labelled entities may be measured by measuring the interaction of the fluorescent labels when in close proximity to each other.

In a fourth aspect, the invention provides a compound which is identifiable by the methods according to the third aspect of the invention.

In a fifth aspect, the invention provides a method of reducing the blood glucose level of a mammalian animal comprising administering a therapeutically effective amount of an inhibitor compound as defined in accordance with the previous aspects of the invention. Preferably the mammalian animal is a human.

By "therapeutically effective amount" we include the meaning that enough of the compound is administered to produce a beneficial effect in the recipient, for example a beneficial decrease in hyperglycaemia.

A sixth aspect of the invention provides a method of identifying a compound which mimics the effect of phosphorylase *a* on G_L, the method comprising contacting said compound with G_L and determining whether, in the presence of the compound, G_L adopts the properties of G_L in the presence of phosphorylase *a*.

By "mimics the effect of phosphorylase *a*" we include the meaning that the compound modifies a property of G_L in such a way that G_L acts, in at least one respect, like G_L that is interacting with phosphorylase *a*.

It will be appreciated that the G_L may be bound to PP1c, ie may be in the form of PP1G_L and that the effect of the compound may be assessed by measuring the activity of PP1G_L, as well known to those skilled in the art, for example as described in WO97/37224 and references therein. Thus, the dephosphorylation of glycogen synthase by PP1G_L may be measured. Thus a compound may be selected that decreases the activity of PP1G_L. It will be appreciated that the method may include the conduct of a screen or screens to determine that the compound interacts with the G_L subunit and not with the PP1c subunit.

The said compound may be a drug-like compound or lead compound for the development of a drug-like compound. Thus, the method may be a method for identifying a drug-like compound or lead compound for the

development of a drug-like compound that is capable of mimicking the effect of phosphorylase α on G_L (a mimic compound).

In a seventh aspect, the invention provides a compound which is identifiable by the methods according to the sixth aspect of the invention.

In an eighth aspect, the invention provides a method of increasing the blood glucose level of a mammalian animal comprising administering a therapeutically effective amount of an inhibitor compound as defined in accordance with the previous aspects of the invention. Preferably the mammalian animal is a human. The mammalian animal, for example human, may have hypoglycaemia.

By "therapeutically effective amount" we include the meaning that enough of the compound is administered to produce a beneficial effect in the recipient, for example a beneficial decrease in hypoglycaemia.

A further aspect of the invention provides a kit of parts useful in carrying out the method of the third aspect of the invention. Thus such a kit may comprise phosphorylase α and a polypeptide comprising the sequence of the C-terminal 16 amino acids of human G_L sequence, or a fragment or variant thereof of which is capable of binding phosphorylase α , for example the 16 amino acid sequence PEWPSYLGYEKLGYYY, or a fragment or variant thereof which binds phosphorylase α .

Preferred embodiments of the invention will now be described by way of example, with reference to the accompanying figures.

Figure 1. Schematic representation of the G_L and truncated forms generated by PCR or restriction digestion. The ability of G_L and the G_L fragments to bind PP1, glycogen and phosphorylase α is indicated. + indicates that binding was observed; - denotes that binding was tested but not detected; a indicates that aggregation of G_L and its derivatives precluded testing of glycogen binding; interactions that were not tested are left blank.

Figure 2. Identification of the G_L region required for interaction with glycogen. GST- G_L fusion proteins containing truncated G_L coding regions were examined for their ability to co-sediment with glycogen as described hereinafter. The supernatant (S) and the pellet (P) fractions obtained in the absence and presence of glycogen were subjected to SDS/PAGE on 12.5% polyacrylamide gels, transferred to nitrocellulose membranes and immunoblotted with affinity purified anti- G_L protein antibodies. The position of the standard marker proteins, glycogen phosphorylase (97kDa), bovine serum albumin (66kDa), ovalbumin (43kDa), and carbonic anhydrase (30kDa) are indicated.

Figure 3. Identification of the binding site for phosphorylase α lies in the 16 carboxy-terminal amino acids of G_L . GST- G_L (1-284) and GST-fusions containing truncated G_L coding regions (2 μ g) were separated on 12.5% SDS-polyacrylamide gels and either (A) stained with Coomassie blue or (B) transferred to nitrocellulose and probed with 100 nM 32 P-labelled phosphorylase α . The standard marker proteins are as in Fig. 2.

Figure 4. Identification of the region binding PP1 in G_L . GST- G_L (1-284) and GST-fusions containing truncated G_L coding regions (2 μ g) were

separated on 12.5% SDS-polyacrylamide gels and either (A) stained with Coomassie blue or (B) transferred to nitrocellulose and probed with Digoxigenin-labelled PP1 γ . The standard marker proteins are as in Fig. 2.

Figure 5. Comparison of the sequences of rat M₁₁₀ 1-38 peptide and rabbit G_M 63-93 peptide with G_L in the region of the RVSF motif (underlined). Three basic residues (Lys or Arg) preceding the RVSF motif that are conserved in M₁₁₀ and G_L but not present in the G_M 63-93 peptide are double underlined. No other clear sequence similarities between M₁₁₀ 1-38 peptide and G_L were apparent in the region preceding that shown.

Figure 6. Comparison of the polysaccharide binding domain of the mammalian glycogen targeting subunits, *S. cerevisiae* GAC1, *Rhizopus oryzae* glucoamylase (AMYL) with phosphorylase (PHOS) glycogen binding (storage) site. The sequences are rat G_L[5], human PPP1R5[6], human PPP1R6[7], human G_M[4], *S. cerevisiae* GAC1[19], *R. oryzae* glucoamylase [20] and rabbit skeletal muscle glycogen phosphorylase [21]. A consensus sequence for the polysaccharide binding domain of the glycogen targeting subunits and glucoamylase is shown. Conserved residues are underlined and identical residues are double underlined. The maltohepatose binding residues in phosphorylase are marked with an asterisk.

Figure 7. Schematic representation of the domains in G_L that interact with PP1, glycogen and phosphorylase α . PP1 binds to the Arg-Val-Ser-Phe motif located at residues 61-64. The basic sequence preceding this

motif is also likely to be involved in binding PP1. Residues 134-231 include the conserved residues among the glycogen subunits that lie in the 94-257 fragment of G_L that co-sediments with glycogen. Phosphorylase α binds to the carboxyterminal terminal 16 amino acids of G_L . The relevant amino acid positions in G_L are indicated above.

MATERIALS AND METHODS

1. *Production of glutathione S-transferase- G_L fusion proteins*

The pGEX- G_L construct containing the complete coding region of G_L [5] was used as a template in polymerase chain reactions using primer pairs to generate a variety of G_L coding region fragments (Fig. 1). The 5' coding primers all contained an *NdeI* site and the 3' reverse primers contained the termination codon and a *XhoI* site as described in [5]. The PCR products were then subcloned into the TOPO 2.1 PCR cloning vector (Invitrogen, Leek, The Netherlands) and verified by sequencing on an Applied Biosystems 373A automated DNA sequencer using Taq dye terminator cycle sequencing. The G_L coding region fragments were subsequently excised by restriction cleavage with *NdeI* and *XhoI* and ligated into the pGEX-AH vector digested with the same restriction enzymes. The G_L 1-94 and 1-170 truncations were generated by excision of a *SacI-SacI* and a *HindIII-HindIII* restriction fragment respectively from the pGEX- G_L construct followed by religation of the plasmid. The G_L 94-170 truncation was generated by *NdeI-HindIII* digestion of pGEX- G_L 94-257 followed by ligation of the fragment into pGEX-AH vector digested with the same enzymes. Site directed mutation of the single mutants N152A, K157A, the double mutant N152A + K157A and the triple mutant K149A +

N152A + L153A were generated as described in [14] using pGEX-G_L 94-257 as template. The resultant constructs encoded glutathione S-transferase (GST) fused to G_L coding region fragments of various lengths. Soluble GST-G_L fusion-proteins were obtained by growing *E. coli*, transformed with the various pGEX-G_L deletion constructs, in LB medium containing 100 µg/ml ampicillin and inducing expression in log phase growth at an A_{600nm} of 0.5 with 0.2 mM isopropyl-thio-β-D-galactopyranoside. After 16 hours further growth at 26°C-28°C, the *E. coli* were harvested and soluble GST-G_L fusion proteins were purified in glutathione agarose as described in [5].

2. *Interaction of GST-G_L fusion proteins with digoxigenin-PPI and phosphorylase a*

GST-G_L fusion proteins were separated on SDS-PAGE and transferred to nitrocellulose membranes. They were probed with digoxigenin-PPI as described [7]. Alternatively they were examined for binding of ³²P-labelled phosphorylase a. Non-specific binding to the membranes was blocked by incubation in 5% (w/v) marvel dried milk powder, 25 mM Tris/HCl pH 7.5, 500 mM NaCl for 16 hours. The samples were then probed for 3 h with ³²P-labelled phosphorylase a (100 nM) in 25 mM Tris/HCl pH 7.5, 250 mM NaCl, 1 mg/ml bovine serum albumin. The membranes were subsequently washed (3 x 30 min) with 25 mM Tris/HCl pH 7.5 before autoradiography.

3. Co-sedimentation of GST- G_L fusion proteins with hepatic glycogen

Protein free glycogen was prepared by the following protocol. Glycogen-protein particles were isolated from the livers of New Zealand White rabbits [15]. Protein was then stripped from the glycogen by boiling for 5 min in 1 % (w/v) sodium dodecyl sulphate (SDS). The suspension was cooled to room temperature and centrifuged for 60 min at 100,000 \times g. The 100,000 \times g pellet was then resuspended in water, and the centrifugation and resuspension procedure repeated a further two times in order to completely remove residual SDS and protein. Any contaminating nucleotides were removed by incubating the glycogen for 15 min with mixed bed resin, AG 501-X8(D). The resin was removed by filtration and the glycogen concentration determined by the phenol/sulphuric acid method [16].

Protein free glycogen (10 mg/ml) in 50 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.1 % (v/v) 2-mercaptoethanol, 0.02% (w/v) Brij-35, 0.1 mg/ml bovine serum albumin was mixed with GST-GL fusion proteins (50 nM). After incubation on ice for 30 min, the samples were centrifuged for 90 min at 100,000 \times g. The supernatant and pellet fractions were denatured in SDS, subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The membranes were incubated overnight in 25 mM Tris/HCl pH 7.5, 250 mM NaCl, 0.1 % (w/v) Tween-20, 10% dried milk powder before probing with affinity purified sheep anti-GL protein antibodies (100 ng/ml in 25 mM Tris/HCl pH 7.5, 250 mM NaCl, 0.1% (w/v) Tween-20, 3% (w/v) dried milk powder), followed by several washes in the same buffer (without the dried milk powder) and incubation with horse radish peroxidase-conjugated anti-sheep antibodies (Pierce,

UK). Immunoreactive bands were visualised using the enhanced chemiluminescence system (Amersham International, Bucks, UK.)

4. Results

4.1 *Residues 94-257 of G_L are required for binding to glycogen*

GST-G_L, containing the full-length G_L coding region, and several of the GST-G_L truncations {GST-G_L(94-216), GST-G_L(94-170), GST-G_L(134-170), GST-G_L(134-216), GST-G_L(134-257)} exhibited a strong tendency to aggregate and were pelleted at 100 000 x g for 1 h, even in the absence of glycogen. Therefore these constructs could not be tested for glycogen dependent sedimentation. Of the GST-fusion proteins that did not aggregate, GST-G_L(94-284) and GSTG_L(94-257) were both detected exclusively in the 100,000 x g pellet obtained by centrifugation in the presence of glycogen (Fig. 2). In contrast GST-G_L(170-216) and GST-G_L(170-257) did not bind to glycogen, being detected exclusively in the 100,000 x g supernatant fraction in the presence of glycogen (Fig. 2). GST-G_L(94-257) carrying the single mutations N152A or K157A, the double mutation N152A + K157A, or the triple mutation K149A + N152A + L153A were all found to sediment in the presence of glycogen (data not shown).

4.2 *The phosphorylase a binding site lies in the C-terminal 16 amino acids of G_L*

In order to identify the region of G_L that is responsible for the binding of phosphorylase α , GST-G_L and its truncated forms were transferred to

nitrocellulose membranes and tested for their ability to bind 32 P-phosphorylase *a*. 32 P-labelled phosphorylase *a* was found to bind to GST- G_L containing the entire G_L coding region and to GST- G_L (216-284), GST- G_L (257-284) and GST- G_L (269-284) but not to GST- G_L (1-216), GST- G_L (1-257) and GST- G_L (1-271) {Fig. 3 and data not shown). These results indicate that the phosphorylase *a* binding domain lies in the carboxy-terminal 16 amino acids of G_L .

4.3 *The PPI-binding domain lies between residues 59 and 94 of G_L*

The GST- G_L truncations were also tested for their ability to bind digoxigenin-labelled PP1 after transfer to nitrocellulose membranes. Fig. 4 shows that digoxigenin-PP1 binds to GST- G_L (1-284), GST- G_L (1-94) and GST- G_L (59-284) but not to GST- G_L (1-59) or GST- G_L (94-284), GST- G_L (134-284) or GST- G_L (170-284). From these interactions, the principal PP1 binding domain must lie between residues 59 and 94 of G_L . Several proteolytically degraded fragments present in the preparations were also recognised by digoxigenin-PP1, in particular a minor 35 kDa Coomassie-blue staining band that migrated slightly faster than GST- G_L (1-94) but slower than GST- G_L (1-59). Since this proteolytic fragment was retained on glutathione-Sepharose, it is likely to comprise GST linked to the first 75-80 residues of G_L . The strong signal with digoxigenin-PP1 may be explained by a more effective renaturation of this fragment from SDS on the nitrocellulose membrane.

5. *Discussion*

Here we have identified three distinct functional domains on the rat liver glycogen-targeting subunit of protein phosphatase 1. The section comprising amino acids 59-94 is both necessary and sufficient for binding to PP1. This region contains the sequence, Arg-Val-Ser-Phe which conforms to the consensus PP1-binding motif determined for other PPI-binding subunits. The data provides further evidence for the importance of this short motif in the binding of PP1 to its targeting subunits. The results also indicate that no other domains outside of residues 59-94 can initiate and maintain an interaction with PP1 independently of the RVSF motif. Previous experiments [12] demonstrated that a peptide comprising the amino terminal 38 residues of the M_{110} subunit of the myosin targeting subunit of PP1 (M_{110} 1-38) could disrupt the PP1G_L complex, while a peptide comprising residues 63-93 of the skeletal muscle glycogen targeting subunit (G_M 63-93) did not abolish binding of G_L to PP1. This data suggests that secondary sites of PP1G_L interaction are likely to involve residues that are identical in M_{110} 1-38 and G_L but distinct (or not included) in G_M 63-93. A comparison of the sequences of M_{110} 1-38 peptide and G_M 63-93 peptide with G_L in the region of the RVSF motif identifies 3 basic residues (Lys or Arg) preceding the RVSF motif that are identical in M_{110} and G_L but are not present in the G_M 63-93 peptide (Fig. 5). The basic residues in positions -2, -4 and -5 with respect to the RVSF motif may therefore provide the secondary interactions of G_L with PP1 that are not disrupted by the G_M 63-93 peptide. The crystal structure of PP1 complexed to the G_M 63-75 peptide reveals the presence of an acidic domain in PP1, that ties to the amino terminal end of the RVSF motif in

the bound peptide and thus has the potential to interact with the basic residues in G_L and the M_{110} peptide [2].

The sequence similarity noted previously between the mammalian glycogen targeting subunits and glucoamylase from *Rhizopus oryzae*, which binds starch, spans a region comprising amino acids 134-231 of G_L[5,6] and (Fig. 6). This present study demonstrates that the region 94-257 of G_L is capable of binding to glycogen, while the truncated GST fusion proteins GST-G_L(170-216) or GST-G_L(170-257) fail to bind to glycogen. The interaction of GST-G_L(94-170) or GST-G_L(134-170) with glycogen could not be tested due to the aggregation of this fragment. However, residues 148-168 of G_L show some sequence similarities (Fig. 6) to the region in phosphorylase identified in crystallographic studies to bind to maltoheptaose and believed to bind to glycogen *in vivo* [17, 181]. The crystal structure of phosphorylase shows that the side chains of conserved hydrophobic amino acids in this section point inwards and appear to be involved in maintaining the orientation of the α -helix that binds maltoheptaose. Of the residues shown to bind to maltoheptaose, only the Asn corresponding to Asn152 in G_L is identical in the glycogen targeting subunits and phosphorylase. However, mutation of N152A in GST-G_L(94-257) did not prevent this fragment binding to glycogen, nor did the double mutation of K157A + N152A. The triple mutation K149A + N152A + L153A of residues which are conserved in the glycogen targeting subunits and are aligned with those binding maltoheptaose in phosphorylase (Fig. 6) also did not prevent the binding of GST-G_L(94-257) to glycogen. The results suggest that all of the conserved sections in G_L (134-231) are likely to be required for binding to glycogen either by making direct contact with glycogen or by contributing to structural elements required for this binding. They further indicate that the binding

site of the PP1 glycogen targeting subunit and glucoamylase to polysaccharides is distinct from the glycogen binding (storage) site of phosphorylase.

The allosteric inhibition of the glycogen synthase phosphatase activity of PP1GL by phosphorylase α is mediated by the binding of phosphorylase α to G_L [5,9,11]. The results presented here demonstrate that the 16 amino acids at the C-terminus of G_L are essential for the interaction with phosphorylase α (Fig. 7). However, although this short region is sufficient for the binding of phosphorylase α , it is likely that other regions of G_L are required to transmit the allosteric effect of this molecule to the active site of PP1. Nevertheless, the unexpected identification of a short sequence in G_L as crucial for the binding of phosphorylase α and therefore also for inhibition of glycogen synthase phosphatase activity provides a rationale for searching for small molecules that might block this inhibition. Raising the level of glycogen synthase phosphatase and consequently glycogen synthase may be useful in disorders, such as diabetes, where hyperglycaemia is a severe problem.

The other three glycogen-binding subunits, G_M /PPP1R3, PPP1R5 and PPP1R6 do not show significant sequence similarity to the carboxy-terminus of G_L [7] which explains why G_M is not inhibited by phosphorylase α [10] and PPP1R5 and PPP1R6 have not been found to bind phosphorylase α after transfer to nitrocellulose membranes [5, 7]. PTG, the mouse homologue of the human glycogen-targeting subunit PPP1R5, has been reported to bind to phosphorylase α , as well as to several other regulatory enzymes of glycogen metabolism [8]. If this is the case, then the amino acid sequence binding, phosphorylase α in PTG is significantly different from that in G_L .

6. *Method of identifying an inhibitor compound which is capable of blocking the interaction of phosphorylase α with the glycogen-targeting subunit of PP1.*

GST-G_L forms containing the 16 amino acid sequence [X] produced in accordance with the previously described methods are transferred to nitrocellulose membranes and tested for their ability to bind ³²P-labelled phosphorylase α in the presence and absence of a test compound. A decrease in the amount of ³²P phosphorylase α binding in the presence of the test compound compared to the amount of binding in the absence of the test compound is indicative of an inhibitory compound of the invention.

A compound which is capable of blocking the phosphorylase α G_L interaction should increase the activity of PP1-G_L and consequently that of glycogen synthase, leading to the increased conversion of the glucose into glycogen. Hence, the compound should be an effective drug in lowering blood glucose by converting it into hepatic glycogen.

Previously, it was reasonable to assume that since phosphorylase α is a large molecule, it might bind to many sites or an extensive region of G_L. Consequently, the probability of finding a drug which could block this interaction was thought to be low. The unexpected identification of the binding site on G_L has been only 16 amino acids long (or smaller) increases the likelihood of finding a drug that will prevent phosphorylase α inhibition of the PP1-G_L complex and hence increase glycogen synthesis.

7. *Use in medicine*

The aforementioned compounds of the invention or a formulation thereof may be administered by any conventional method including oral and parenteral (eg subcutaneous or intramuscular) injection. The treatment may consist of a single dose or a plurality of doses over a period of time.

8. *Pharmaceutical compositions of the invention*

The following examples illustrate pharmaceutical formulations according to the invention in which the active ingredient is a compound of the invention.

Example A: Tablet

Active ingredient	100 mg
Lactose	200 mg
Starch	50 mg
Polyvinylpyrrolidone	5 mg
Magnesium stearate	4 mg
	359 mg

Tablets are prepared from the foregoing ingredients by wet granulation followed by compression.

Example B: Tablet Formulations

The following formulations A and B are prepared by wet granulation of the ingredients with a solution of povidone, followed by addition of magnesium stearate and compression.

Formulation A

	<u>mg/tablet</u>	<u>mg/tablet</u>
(a) Active ingredient	250	250
(b) Lactose B.P.	210	26
(c) Povidone B.P.	15	9
(d) Sodium Starch Glycolate	20	12
(e) Magnesium Stearate	5	3
	—	
	500	300

Formulation B

	<u>mg/tablet</u>	<u>mg/tablet</u>
(a) Active ingredient	250	250
(b) Lactose	150	-
(c) Avicel PH 101 [*]	60	26
(d) Povidone B.P.	15	9
(e) Sodium Starch Glycolate	20	12
(f) Magnesium Stearate	5	3
	—	
	500	300

Formulation C

	<u>mg/tablet</u>
Active ingredient	100
Lactose	200
Starch	50
Povidone	5
Magnesium stearate	4

359

The following formulations, D and E, are prepared by direct compression of the admixed ingredients. The lactose used in formulation E is of the direct compression type.

Formulation D

	<u>mg/capsule</u>
Active Ingredient	250
Pregelatinised Starch NF15	150
	400

Formulation E

	<u>mg/capsule</u>
Active Ingredient	250
Lactose	150
Avicel [®]	100
	500

Formulation F (Controlled Release Formulation)

The formulation is prepared by wet granulation of the ingredients (below) with a solution of povidone followed by the addition of magnesium stearate and compression.

	<u>mg/tablet</u>
(a) Active Ingredient	500
(b) Hydroxypropylmethylcellulose (Methocel K4M Premium) [®]	112
(c) Lactose B.P.	53
(d) Povidone B.P.C.	28
(e) Magnesium Stearate	7
	700

Drug release takes place over a period of about 6-8 hours and was complete after 12 hours.

Example D: Capsule FormulationsFormulation A

A capsule formulation is prepared by admixing the ingredients of Formulation D in Example C above and filling into a two-part hard gelatin capsule. Formulation B (*infra*) is prepared in a similar manner.

Formulation B

	<u>mg/capsule</u>
(a) Active ingredient	250
(b) Lactose B.P.	143
(c) Sodium Starch Glycolate	25
(d) Magnesium Stearate	2
	420

Formulation C

	<u>mg/capsule</u>
(a) Active ingredient	250
(b) Macrogol 4000 BP	350
	600

Capsules are prepared by melting the Macrogol 4000 BP, dispersing the active ingredient in the melt and filling the melt into a two-part hard gelatin capsule.

Formulation D

	<u>mg/capsule</u>
Active ingredient	250
Lecithin	100
Arachis Oil	100
	450

Capsules are prepared by dispersing the active ingredient in the lecithin and arachis oil and filling the dispersion into soft, elastic gelatin capsules.

Formulation E (Controlled Release Capsule)

The following controlled release capsule formulation is prepared by extruding ingredients a, b, and c using an extruder, followed by spheronisation of the extrudate and drying. The dried pellets are then coated with release-controlling membrane (d) and filled into a two-piece, hard gelatin capsule.

	<u>mg/capsule</u>
(a) Active ingredient	250
(b) Microcrystalline Cellulose	125
(c) Lactose BP	125
(d) Ethyl Cellulose	13

513

Example E: Injectable Formulation

Active ingredient 0.200 g

Sterile, pyrogen free phosphate buffer (pH7.0) to 10 ml

The active ingredient is dissolved in most of the phosphate buffer (35-40°C), then made up to volume and filtered through a sterile micropore filter into a sterile 10 ml amber glass vial (type 1) and sealed with sterile closures and overseals.

Example F: Intramuscular injection

Active ingredient	0.20 g
Benzyl Alcohol	0.10 g
Glucofuro 75°	1.45 g
Water for Injection q.s. to	3.00 ml

The active ingredient is dissolved in the glycofuro. The benzyl alcohol is then added and dissolved, and water added to 3 ml. The mixture is then filtered through a sterile micropore filter and sealed in sterile 3 ml glass vials (type 1).

Example G: Syrup Suspension

Active ingredient	0.2500 g
Sorbitol Solution	1.5000 g
Glycerol	2.0000 g
Dispersible Cellulose	0.0750 g
Sodium Benzoate	0.0050 g
Flavour, Peach 17.42.3169	0.0125 ml
Purified Water q.s. to	5.0000 ml

The sodium benzoate is dissolved in a portion of the purified water and the sorbitol solution added. The active ingredient is added and dispersed. In the glycerol is dispersed the thickener (dispersible cellulose). The two dispersions are mixed and made up to the required volume with the purified water. Further thickening is achieved as required by extra shearing of the suspension.

Example H: Suppositorymg/suppository

Active ingredient (63 μ m)*	250
Hard Fat, BP (Witepsol H15 - Dynamit Nobel)	1770

2020

*The active ingredient is used as a powder wherein at least 90% of the particles are of 63 μ m diameter or less.

One fifth of the Witepsol H15 is melted in a steam-jacketed pan at 45°C maximum. The active ingredient is sifted through a 200 μ m sieve and added to the molten base with mixing, using a silverson fitted with a cutting head, until a smooth dispersion is achieved. Maintaining the mixture at 45°C, the remaining Witepsol H15 is added to the suspension and stirred to ensure a homogenous mix. The entire suspension is passed through a 250 μ m stainless steel screen and, with continuous stirring, is allowed to cool to 40°C. At a temperature of 38°C to 40°C 2.02 g of the mixture is filled into suitable plastic moulds. The suppositories are allowed to cool to room temperature.

Example I: Pessaries

	<u>mg/pessary</u>
Active ingredient	250
Anhydrate Dextrose	380
Potato Starch	363
Magnesium Stearate	7
	1000

The above ingredients are mixed directly and pessaries prepared by direct compression of the resulting mixture.

The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient (compound of the invention) with the carrier which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Formulations in accordance with the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets, each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented as a bolus, electuary or paste.

A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder (eg povidone, gelatin, hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (eg sodium starch glycolate, cross-linked povidone, cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Moulded tablets may be made by moulding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active

ingredient therein using, for example, hydroxypropylmethylcellulose in varying proportions to provide desired release profile.

Formulations suitable for topical administration in the mouth include lozenges comprising the active ingredient in a flavoured basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouth-washes comprising the active ingredient in a suitable liquid carrier.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Preferred unit dosage formulations are those containing a daily dose or unit, daily sub-dose or an appropriate fraction thereof, of an active ingredient.

It should be understood that in addition to the ingredients particularly mentioned above the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question.

for example those suitable for oral administration may include flavouring agents.

Whilst it is possible for a compound of the invention to be administered alone, it is preferable to present it as a pharmaceutical formulation, together with one or more acceptable carriers. The carrier(s) must be "acceptable" in the sense of being compatible with the compound of the invention and not deleterious to the recipients thereof. Typically, the carriers will be water or saline which will be sterile and pyrogen free.

Claims

1. Use in medicine of a compound which is capable of blocking the interaction of phosphorylase α with the glycogen-targeting subunit (G_L) of protein phosphatase 1.
2. Use as claimed in Claim 1 wherein the compound is for use in the manufacture of a medicament for use in the treatment of a disorder associated with higher than normal blood glucose levels.
3. Use as claimed in Claim 2 wherein the disorder is selected from type I and/or type II diabetes.
4. Use in medicine as claimed in Claim 1 wherein the compound is a polypeptide comprising the 16 mer amino acid sequence PEWPSYLGYEKLYPYY, or a fragment or variant thereof which is capable of binding phosphorylase α .
5. Use as claimed in Claim 4 wherein the polypeptide increases the activity of hepatic glycogen synthase.
6. A pharmaceutical composition comprising an inhibitor compound which is capable of blocking the interaction of phosphorylase α with the glycogen - targeting subunit (G_L) of protein phosphatase 1, together with a pharmaceutically acceptable excipient or carrier.
7. A pharmaceutical composition as claimed in Claim 6 wherein the inhibitor compound comprises a polypeptide having the 16 mer amino acid

sequence PEWPSYLGYLEKLYPYY, or a fragment or variant thereof which is capable of binding phosphorylase α .

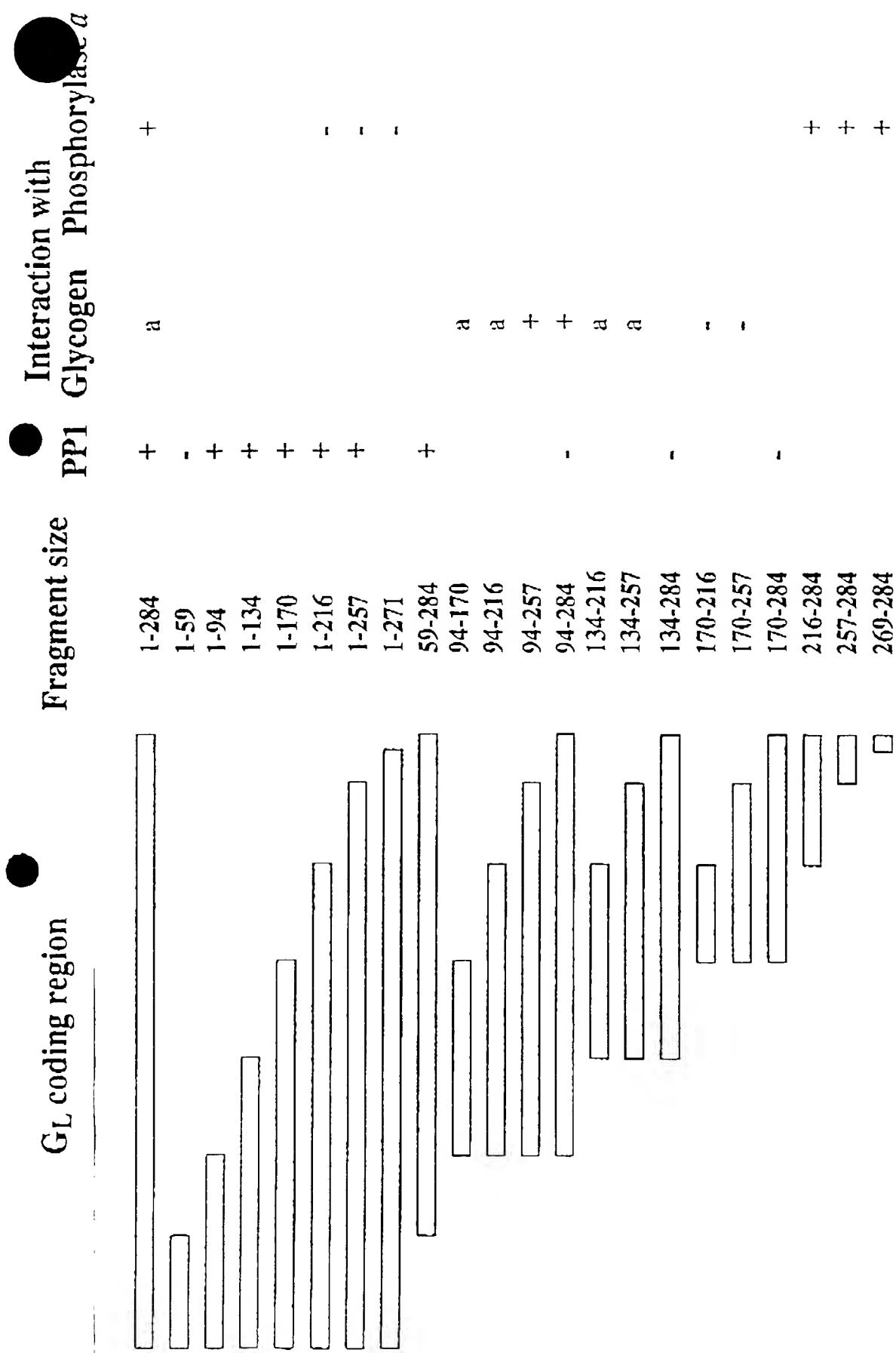
8. A pharmaceutical composition as claimed in Claim 6 wherein the polypeptide consists of a truncated version of the glycogen-targeting subunit of protein phosphatase 1.
9. A method of identifying an inhibitor compound which is capable of blocking the interaction of phosphorylase α with the glycogen-targeting subunit of protein phosphatase 1 comprising;
providing a polypeptide comprising the 16 mer amino acid sequence PEWPSYLGYLEKLYPYY, or fragment or variant thereof which binds phosphorylase α ;
providing a test compound; and
comparing the binding of the polypeptide by phosphorylase α in the presence or absence of the test compound; an inhibitor being identified by reduced binding in the presence of the test compound.
10. A method as claimed in Claim 9 wherein the phosphorylase α is labelled and the binding of phosphorylase α to the polypeptide is determined by measuring the amount of label.
11. A method as claimed in Claim 10 wherein phosphorylase α is labelled with a label selected from digoxigenin and ^{32}P or ^{33}P .
12. A compound which is identifiable by the method of any one of Claims 9 to 11.

13. A method of reducing the blood glucose level of a mammalian animal comprising administering a therapeutically effective amount of an inhibitor compound as defined in any one of Claims 1 to 12.
14. A method as claimed in Claim 13 wherein the mammalian animal is a human.

ABSTRACT

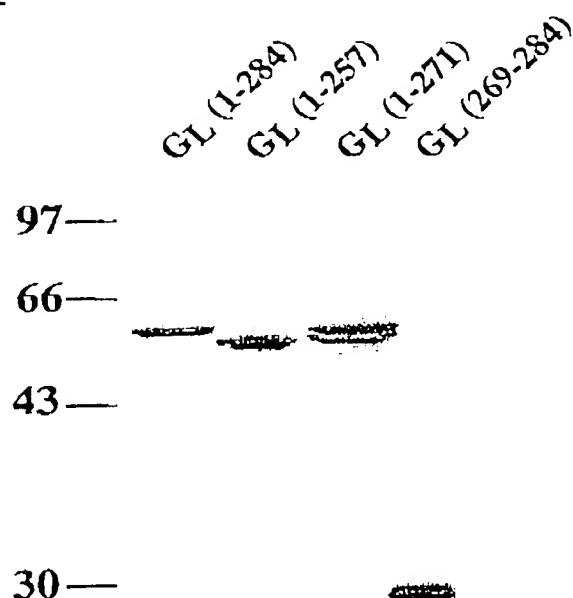
Therapeutic compounds and methods of making and using the same.
The present invention relates to compounds useful for treating disorders associated with abnormal blood glucose levels in mammals, such as Type I and Type II diabetes. The invention also relates to methods of making and using such compounds.

FIG. 7

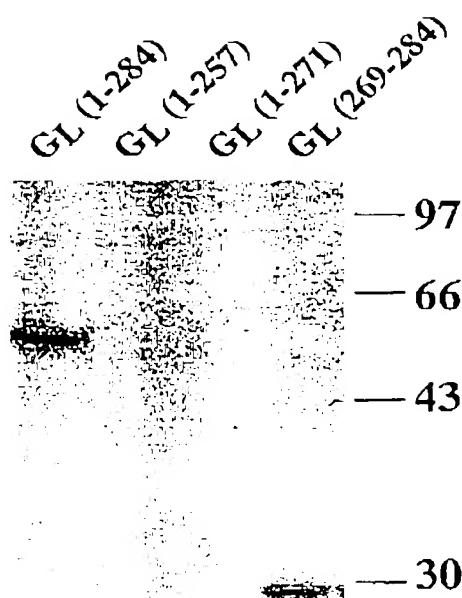


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M ₁₁₀ (1-38) peptide	26	... PPVV <u>KRQKTKVKE</u>	38
G _L	52	... TVQE <u>KKVKKRVSFADQGL</u> ...	69
G _M (63-93) peptide	63	G <u>RRVSFADNFG</u> ...	73

Fig 5

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G _L	132	V <u>C</u> L <u>E</u> ---NCVL <u>K</u> E <u>K</u> A <u>L</u> A <u>G</u> T <u>V</u> K <u>V</u> N <u>L</u> A <u>F</u> E <u>K</u> V <u>V</u> K <u>I</u> R <u>M</u> -- <u>T</u> <u>F</u> D <u>T</u> <u>W</u> K <u>S</u> F <u>T</u>	172
R ₅	157	V <u>C</u> L <u>E</u> ---N <u>C</u> S <u>L</u> Q <u>E</u> R <u>T</u> V <u>T</u> G <u>T</u> V <u>K</u> V <u>N</u> S <u>F</u> E <u>K</u> V <u>Q</u> I <u>R</u> I-- <u>T</u> <u>F</u> D <u>S</u> <u>W</u> K <u>N</u> Y <u>T</u>	197
R ₆	177	V <u>C</u> L <u>E</u> ---R <u>V</u> T <u>C</u> S <u>D</u> L <u>G</u> I <u>S</u> G <u>T</u> V <u>R</u> V <u>C</u> N <u>V</u> A <u>F</u> E <u>K</u> Q <u>A</u> V <u>R</u> Y-- <u>T</u> <u>F</u> S <u>G</u> W <u>R</u> S <u>T</u> H	217
G _M	128	A <u>I</u> L <u>E</u> S-T <u>E</u> S <u>L</u> L <u>G</u> S <u>T</u> S <u>I</u> K <u>G</u> I <u>I</u> R <u>V</u> L <u>N</u> V <u>S</u> F <u>E</u> K <u>L</u> V <u>V</u> R <u>M</u> -- <u>S</u> <u>L</u> D <u>D</u> W <u>Q</u> T <u>H</u> Y	170
GAC1	244	V <u>K</u> L <u>H</u> S <u>L</u> T <u>Q</u> L <u>G</u> D <u>D</u> S <u>S</u> K <u>I</u> T <u>G</u> L <u>V</u> V <u>K</u> N <u>L</u> S <u>F</u> E <u>K</u> Y <u>L</u> E <u>I</u> K <u>F</u> -- <u>T</u> <u>F</u> N <u>S</u> <u>W</u> R <u>D</u> I <u>H</u>	287
AMYL	33	V <u>O</u> L <u>D</u> S---Y <u>N</u> Y <u>D</u> G <u>S</u> T <u>F</u> S <u>G</u> K <u>I</u> Y <u>V</u> K <u>N</u> I <u>A</u> Y <u>S</u> K <u>V</u> T <u>V</u> I <u>Y</u> A <u>D</u> G <u>S</u> D <u>N</u> W <u>N</u> N <u>N</u> G	75
Consensus		Φ. G. V. V. NΦ. F. K. V. V. Φ. W I Y L I	

PHOS	398	R <u>H</u> L <u>Q</u> I <u>I</u> Y <u>E</u> I <u>N</u> Q <u>R</u> E <u>L</u> N <u>R</u> V <u>A</u> A <u>F</u> P <u>G</u> D <u>V</u> D <u>R</u> L <u>R</u> R <u>M</u> S	429
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G _L	173	D <u>F</u> P <u>C</u> Q <u>Y</u> V <u>K</u> D <u>T</u> Y <u>A</u> G <u>S</u> D <u>R</u> D <u>T</u> F <u>S</u> <u>F</u> D <u>I</u> S <u>L</u> P <u>E</u> K <u>I</u> Q <u>S</u> Y <u>E</u> -----	205
R ₅	198	D <u>V</u> D <u>C</u> V <u>Y</u> M <u>K</u> N <u>V</u> Y <u>G</u> G <u>T</u> D <u>S</u> D <u>T</u> F <u>S</u> <u>F</u> A <u>I</u> D <u>L</u> P <u>P</u> V <u>I</u> P <u>T</u> E <u>Q</u> -----	230
R ₆	218	E <u>A</u> V <u>A</u> R <u>W</u> R <u>G</u> P <u>A</u> G <u>P</u> E <u>G</u> T <u>E</u> D <u>V</u> F <u>T</u> G <u>F</u> P <u>V</u> P <u>P</u> F <u>L</u> L <u>E</u> G <u>S</u> -----	251
G _M	171	D <u>I</u> L <u>A</u> E <u>Y</u> V <u>P</u> N <u>S</u> C <u>D</u> G-E <u>T</u> D <u>O</u> F <u>S</u> E <u>K</u> I <u>V</u> L <u>V</u> P <u>P</u> Y <u>Q</u> K <u>D</u> G <u>S</u> -----	203
GAC1	288	Y <u>V</u> T <u>A</u> N <u>F</u> N <u>R</u> T <u>I</u> N--S <u>N</u> V <u>D</u> E <u>F</u> E <u>T</u> I <u>D</u> L <u>N</u> S <u>K</u> Y <u>I</u> L <u>L</u> I <u>K</u> R <u>I</u> I <u>T</u> M <u>E</u> K <u>N</u> T <u>S</u>	331
AMYL	76	N <u>T</u> I <u>A</u> A <u>S</u> Y <u>S</u> A <u>P</u> I <u>S</u> G <u>S</u> N <u>Y</u> E <u>Y</u> W <u>T</u> F <u>S</u> A <u>S</u> I <u>N</u> G <u>I</u> K-----	104
Consensus		D. F. F. . . Φ E W	

PHOS	330	L <u>V</u> E <u>E</u> G <u>A</u> V <u>K</u>	437
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G _L	206	---R <u>M</u> E <u>F</u> A <u>V</u> C <u>Y</u> E <u>C</u> N <u>G</u> Q <u>S</u> Y <u>W</u> D <u>S</u> N <u>K</u> G <u>K</u> N <u>Y</u> R <u>I</u>	231
R ₅	231	---K <u>I</u> E <u>F</u> C <u>I</u> S <u>Y</u> H <u>A</u> N <u>Q</u> V <u>F</u> W <u>D</u> N <u>N</u> D <u>G</u> Q <u>N</u> Y <u>R</u> I	256
R ₆	252	---R <u>V</u> H <u>F</u> A <u>V</u> R <u>Y</u> Q <u>V</u> A <u>G</u> A <u>E</u> Y <u>W</u> D <u>N</u> N <u>D</u> H <u>R</u> D <u>Y</u> S <u>L</u>	282
G _M	204	---K <u>V</u> E <u>F</u> C <u>I</u> R <u>Y</u> E <u>T</u> S <u>V</u> G <u>T</u> F <u>W</u> S <u>N</u> N <u>N</u> G <u>T</u> N <u>Y</u> T <u>F</u>	229
GAC1	332	C <u>P</u> L <u>N</u> I <u>E</u> L <u>C</u> C <u>R</u> Y <u>D</u> V <u>N</u> N <u>E</u> T <u>Y</u> Y <u>D</u> N <u>N</u> N <u>G</u> K <u>N</u> Y <u>H</u> L	360
AMYL	105	---E <u>F</u> Y <u>I</u> K <u>Y</u> E <u>V</u> S <u>G</u> K <u>T</u> Y <u>Y</u> D <u>N</u> N <u>N</u> S <u>A</u> N <u>Y</u> Q <u>V</u>	128
Consensus		F. Φ. Y. Y. W. . . N. . . . Y. Φ L F Y	

Fig 6

7/7

1 56 64 94 134 231 257 269 284

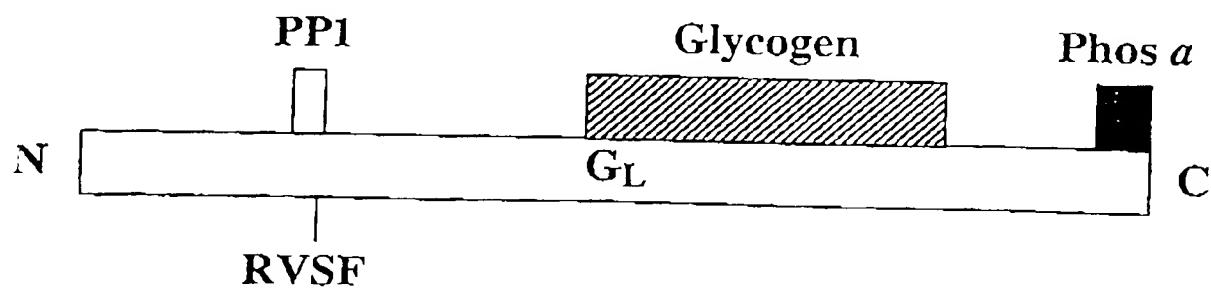


Fig 7

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